Efficacy of a Commercially Available LH Surge Detection Test Strip in the Domestic Bitch.

Honors Research Thesis

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ABSTRACT

Breeding management in the domestic bitch is often utilized in cases of infertility, when the male is not available, or if semen quantity is limited. The process is important but can be costly. Often, endocrine testing will be performed to estimate the timing of ovulation via serum progesterone levels or luteinizing hormone (LH) to accurately determine the optimal timing for insemination. Because of the cost, inconvenience, and stress on the dog, many commercially available tests are available, acting as potential substitutes for conventional methods. It is questionable whether these methods have any accuracy, as research is often limited. One such method includes test strips marketed to detect the presence of the LH surge by measuring the hormone in vaginal secretions. However, our preliminary data show these strips respond positively to glucose solutions regardless of LH serum presence. Research performed in women indicate vaginal glucose may have a correlation with ovulation, but this is unknown to be true in the domestic bitch. Our hypothesis is that the reliability of the commercial LH strips will not compare to that of the techniques widely used by veterinarians today. Our study will evaluate the reproductive cycles of a population of bitches (n=10) with traditional methods in order to determine the LH surge, so that one can predict the time of ovulation and maximum fertility. As part of a clinical trial conducted at the OSU Veterinary Medical Center, we will evaluate hormonal levels of bitches on the days leading up to and following ovulation. We will also monitor glucose concentrations of vaginal fluid during the periovulatory period. Results will be compared across tests to see if commercially available LH strips can be effective for detection of the LH surge, and to determine whether there is a change in vaginal glucose throughout the periovulatory period.

INTRODUCTION

It is extremely beneficial to understand in great detail, the intricacies of the reproductive processes of the domestic bitch for the purposes of breeding management. Overcoming infertility, or artificially inseminating an animal can be quite costly, so researchers have been experimenting with new, efficient methods of detecting ovulation that will be much more convenient and economical for the owner. It is essential to understand the reproductive, or estrous, cycle of the canine. Estrus occurs every 4-24 months, averaging 6 months, and consists of four stages [1]. The first stage, called proestrus, is identified when the bitch begins displaying signs and behaviors of estrous, indicating her receptivity to the male. This initial stage is commonly known as "heat" and is marked by a discernible bloody vulvar discharge. During proestrus, there will also be visible swelling of the vulva. This in combination with discharge, pheromones in the urine, and the different scent of the bitch draws the attention of the males; however, she will not yet allow mounting or breeding at this time. The evident physical changes are triggered by rising concentrations of serum estradiol as the ovarian follicles mature. Proestrus can last from 3 to 21 days, averaging about 9 days [2].

The second stage of the cycle is estrus. Estrus is triggered by a surge in luteinizing hormone (LH) from the pituitary gland prompted by a decrease in estradiol from its peak value [2]. This stage is also considered standing heat. Ovulation occurs approximately two days post LH surge. It is during this time, the bitch is most fertile and receptive to mating. Receptivity can be noted by changes in the vulvar discharge, initially noted within proestrus. It will typically become more "straw-colored" and the vulva will soften, all symptoms making her more receptive to breeding. Unlike other animals that release an oocyte capable of fertilization, the bitch ovulates and releases an immature oocyte, which will then mature over the course of two days until it is ready to be fertilized [2].

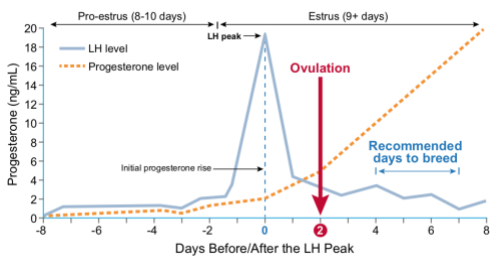

Figure 1. *The trends of progesterone and LH levels during different times of estrus.*

This knowledge of reproductive cyclicity is essential for breeders to determine optimum time for breeding. There are several methods that can be used to analyze where a bitch is currently in her cycle; these can include natural interest of the male, vaginal exfoliative cytology, and serum hormone levels [2]. Many breeders will use male detection dogs to distinguish this stage of optimal breeding because they will show extreme interest in the female and she will permit mounting during estrus. However, these male dogs may be inaccurate or may be unavailable at the time breeding is required. In the case of artificial insemination, or limited availability of the stud, determining the ideal timing for insemination is important. Serum hormone levels are used to help determine estrus and ovulation in the bitch. Progesterone (P4) is a steroid hormone secreted by the developing corpus luteum (CL) on the ovary, and unlike other domestic species, begins to rise prior to ovulation. Measuring serum progesterone is one of the most common ways to estimate ovulation and determine optimum breeding timing. Typically, progesterone begins to rise and reach a level of around 2 ng/ml at the time of the luteinizing hormone (LH) surge. Progesterone will reach levels between 5 and 10 ng/mL on the day of ovulation, followed by a large jump of at least 3 ng/mL in 24 hours [2]. While this information can be very valuable, progesterone levels alone merely estimate ovulation and can be inaccurate when used without supplemental information.

Current methods of estrus detection based on behavioral and physical changes in the bitch do not accurately predict onset of estrus, which is a limitation to reproductive efficiency [3]. The timeline is variable between all types of dogs; behavioral estrus can begin anywhere from 2 days before, to 5 days after the LH peak, so breeding solely based on these factors would not give the best results. Alternative modes of determining the onset of estrus that have been studied include changes in drying patterns of saliva (“ferning”), changes of vaginal pH, and changes of progesterone concentrations in saliva, but none have proved to be as effective as the detection of serum hormone levels [2].

It is known that plasma LH is secreted in a pulsatile way, and its large surge at the time of estrus is what stimulates ovulation of the immature oocyte approximately 36 to 50 hours later [2]. The most predictable method of determining the onset of ovulation, that will supplement the progesterone tests, is to directly measure the presence of luteinizing hormone (LH) in the serum.

There are several commercially available assays to determine this level, as well as a cage-side rapid assay (Witness® LH Test Kit, Zoetis, San Diego, CA), which have been validated for use in the canine. However, this test is costly, stressful on the animal, and requires multiple daily visits to the veterinarian for blood draws to accurately detect estrus. Progesterone assays are often the more reliable method of determining ovulation, but their use also requires multiple blood samples.

In response to this difficulty, several companies market in home LH test strips, which are advertised to change color when exposed to vaginal fluid at the time of the LH surge. However, it is surmised that a change in glucose concentration in vaginal secretion is what triggers the color change when the test strip pad is exposed to the vaginal fluid of the bitch. It has been found that there are, in fact, increased glucose concentrations in the vaginal fluid of the bitch at the onset of estrus [3]. Through our preliminary data, we have identified that the pads will change color in response to the presence of glucose in the vaginal fluid with a minimum threshold of 2.5 mg/mL.

In canines, it is unclear whether vaginal fluid glucose can be used to determine estrus with accuracy, especially due to the differences in reproductive cyclicity across species. In human studies, it was found that there was a good correlation between a glucose strip test and ovulation, but no single test proved consistent [4-6]. Literature regarding glucose presence in canine vaginal fluid is certainly lacking, and some authors believe it is historically inaccurate [7]. Therefore, there appears to be a gap in knowledge as to whether these color changing test strips will accurately predict the LH surge in canines.

These test strips would provide an experimental, non-invasive method of allegedly identifying the LH surge by detecting glucose levels when exposed to the vaginal fluid. Per the manufacturer, a positive result was indicated by a color change of pink to a shade of purple, or the presence of purple specs on and around the border of the detection pad. The strips will be compared to both serum LH levels as determined by radio-labeled immunoassay (RIA) at Colorado State University's veterinary endocrinology laboratory, as well as the standardized Witness® LH Test Kit produced by Zoetis. If successful, this test would give a very practical, cost effective, and efficient mean to accurately determine the best day for insemination in the bitch, limiting the need for frequent blood testing and multiple veterinary visits. It would also be a very practical tool for

the dog breeding industry and allow the opportunity to have the highest breeding potential with much less labor. It is known that the Witness LH test is a reliable method to determine the LH surge in serum and along with qualitative LH values will be used as a "gold standard" in this study [8].

Thus, the objective of the presented study was to determine whether commercially available LH test strips could accurately predict the presence of the LH surge in the bitch. It was hypothesized that the different LH test strips would not be as reliable as the methods widely used amongst veterinarians today. It was also hypothesized that vaginal fluid glucose would not consistently change throughout the periovulatory period in the bitch.

MATERIALS AND METHODS

In order to determine what elicits the color change of the LH test strip, we designed a series of four experiments:

Experiment 1 – Glucose solutions

To test whether or not the test strips changed color in response to glucose within the fluid, the strips were exposed to various concentrations of glucose-containing solutions. Solutions were made by adding glucose to deionized water, exposing the strip for the recommended 30 seconds, and observing for color change. Photos were taken immediately after 30 seconds of exposure.

Concentration	1mg/mL	2mg/mL	2.5mg/mL	5mg/mL
pH	7.23	7.70	8.05	8.14
Observations	No change - pink	No change - pink	Slight change – purple border after 30s-1min	Drastic change – dark purple immediately

Table 1. *LH strips were observed after being exposed to various concentrations of glucose. pH was also recorded at each glucose level.*

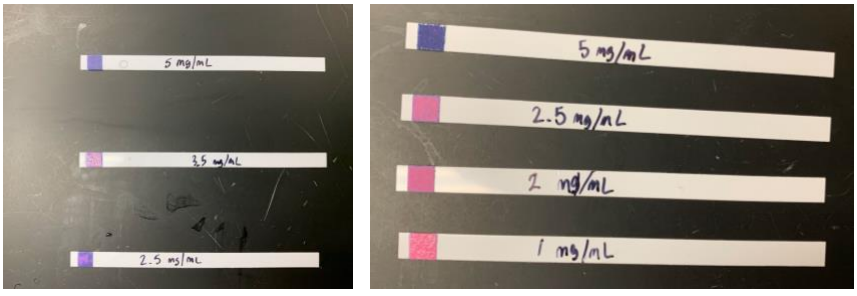

Figure 2. *LH detection strips, labeled with different glucose solution concentrations. Color change was noted at a level of 2.5 mg/mL.*

Experiment 2 – LH solutions (serum)

To test whether the strips changed in response to the presence of LH in the fluid, strips were exposed to serum from an intact bitch (LH-) and a spayed bitch (LH+). To confirm presence of LH within the serum, a Witness LH test was run concurrently. Two lines indicate a positive sample, while one line indicates a negative sample.

Experiment 3 – LH solutions (serum) + glucose

To evaluate whether the glucose concentration of the serum affected the color change of the strip, 2.5mg/ml glucose solution was added to each serum sample.

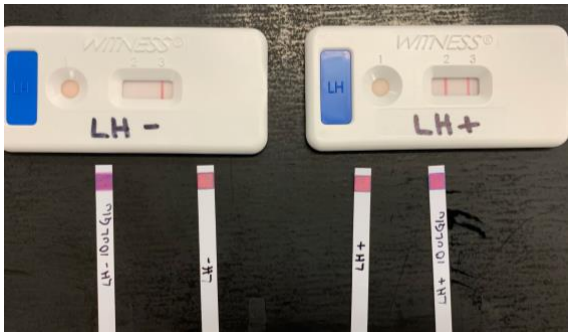

Figure 3. *Two Witness LH tests show a negative and a positive result when exposed to serum from bitches. The LH surge test strips below the tests, with and without added glucose, do not exhibit color change that would be expected when exposed to serum from the LH positive sample.*

Experiment 4 – pH solutions, no glucose

Finally, to evaluate whether the color change was caused by a change in pH of the fluid rather than the addition of glucose, strips were exposed to acidic (phosphate-buffered saline (PBS) + HCl), neutral (PBS), and basic (PBS + NaOH) solutions (pH 2.0, 4.0, 7.0, 9.0) that did not contain glucose.

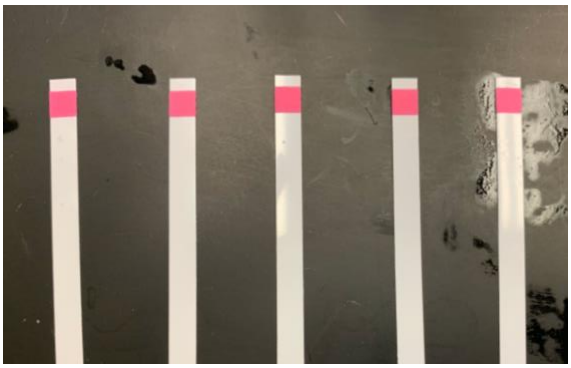

Figure 4. *Five LH detection strips tested at pH levels between 4 and 11. Strips did not change color, indicating a negative result.*

Procedures for the LH study were approved and adhered to the regulations established by the Institutional Animal Care and Use Committee (IACUC). A sample of four sexually mature, client-owned bitches were brought in to The Ohio State University Veterinary Medical Center for routine breeding management. The goal of this study is to evaluate a total of 10 intact bitches with an additional spayed control. We instructed that they be brought in five days after the first sign of visible serosanguinous discharge according to the client, as per typical clinical recommendation.

Client participation was solicited from those that were able to bring their bitches in daily as needed until the LH surge is estimated based on progesterone testing. Blood was drawn at 24 hour intervals and placed into red top tubes. The blood was centrifuged and serum removed. An aliquot of serum was then submitted to the diagnostic laboratory for progesterone (P4) testing, and a second aliquot frozen at -20 C for temporary storage. At each daily visit, the LH Test strip was exposed to the vaginal fluid and evaluated for color change. Per the manufacturer's recommendation, any degree of color change (purple spots, bands, or complete color change) should be regarded as "positive" with no color change indicating a "negative" sample. LH strips that will be tested are the "LH Surge Test Strip" as sold by the vendor, Pet Facets in Marion, NC. A glucose reading of vaginal fluid was also made using an AlphaTRAK® Glucometer validated for determining glucose in canine serum.

Levels of serum progesterone (P4) were tested every 24 to 48 hours until a level was reached correlating with the luteinizing hormone (LH) surge, (approximately 2 ng/mL). Additional samples were taken in the days that follow the theoretical surge, until levels read between 5 and 10 ng/mL, indicating ovulation. Once this P4 level was reached, the serum sample from 2 days prior – the theoretical day of the LH surge – was thawed, and evaluated for presence of LH surge using a commercially available and validated Witness LH test. Additional serum was frozen at -18°C and transferred to -80°C for future submission to the Colorado State University Veterinary Endocrinology Laboratory to acquire quantitative values for plasma LH. These data were used to comparatively determine if the commercial LH test strips can accurately predict optimal breeding time in the bitch. A spayed bitch will serve as a positive control for this experiment due to the persistently elevated LH levels found in circulation in spayed animals at the completion of the study.

The sensitivity, specificity and accuracy of the LH test strips will be determined against the true serum levels of LH. Pearson's correlation will be used to determine the relationship of the LH strips and the Witness LH test once all data is acquired for statistical analysis.

RESULTS

The pilot study consisted of four smaller experiments to determine what the LH strip is detecting. Experiment 1, using only glucose, showed that the color change of the strip intensified as concentration of glucose increased. In experiment 2, no color change was seen in response to only the LH positive or LH negative serum. When glucose was added to the LH serum in experiment 3, color change was noted on both strips in response to the added glucose to the serum, further clarifying that it is not the LH, but glucose that elicited a positive result. Experiment 4 proved that changes in pH do not elicit any color change in the strip, so they are not pH sensitive. Based on this preliminary data, the strips change color intensity as the concentration of glucose increases. A threshold of between 2-2.5 mg/mL of glucose is needed to induce the color change of the strip.

Since this study is ongoing and has faced limitations, we are not yet able to definitively analyze the accuracy of the LH surge test strips. The four subjects currently evaluated all responded to the test strip differently. One bitch had a positive LH strip result that corresponded with the Witness LH test, but the others showed positive or slight positive strip results on the days the Witness test displayed negative. The strips also sometimes showed no color change, a negative result, on the day the Witness test displayed positive (Table 2).

As noted previously in this paper, progesterone rises and reaches a level of around 2 ng/mL at the time of the LH surge [2]. All LH positive samples displayed progesterone levels within a range consistent with this data. Table 2 shows a logical correlation between progesterone levels and the LH surge, as expressed by the Witness test. Subject A had a progesterone level of 1.1 ng/mL on the day she was LH positive. Subject B, C and D had higher progesterone levels of 1.81, 2.1, and 1.6 ng/mL, respectively. All evaluated bitches had LH detected during the expected progesterone interval. Bitches also exhibited flagging behavior within 24 hours of the assumed LH surge.

With the exception of a single sample, vaginal glucose was unable to be detected by the glucometer. Readings of "Er1" or "Lo" were consistent with glucose readings below 20 mg/dL per the manufacturer's guidelines (Table 2).

Table 2. *LH Study Results*

Bitch	*Day*	*P4 (ng/mL)*	*Glucose (mg/dL)*	*LH Strip*	*Witness LH*	*Cytology*
A	1	0.405	23	1		80% cornified
A	2		Lo	1	-	
A	3	1.1	Lo	0	+	
A	4	3.8	Lo	1	Faint +	
A	5		Lo	0		
B	1	< 0.2	Lo	0		>95% cornified
B	2		Lo	0		
B	3	< 0.2	Lo	1		
B	4		Lo	0	-	
B	5	1.81	Er1	2	+	
B	6	2.68	Lo	1	Faint +	
B	7	4.19	Lo	0		
C	1	0.4	Lo	0		>95% cornified
C	2		Lo	0		
C	3	0.2	Er1	2	-	
C	4		Lo	0	Faint +	
C	5	2.1	Er1	0	Faint +	
C	6		Lo	0	-	
C	7	6.1	Lo	0		
D	1	0.3	Er1	0		>95% cornified
D	2		Er1	0		
D	3		Er1	2	-	
D	4	1.6	Er1	1	+	
D	5			0	-	
D	6	5.4	Er1	0		

LH Strip Color Change Key
0 = No change
1 = Borders of pad (+)
2 = Entire pad (+)

DISCUSSION

The goal of this study was to compare the accuracy of a commercially available and affordable LH surge test strip to conventional hormone assays in determining the presence of an LH surge in the domestic bitch. After a series of trials in our pilot study, it was confirmed that the strips did, in fact, have a positive correlation with glucose levels in vaginal fluid. Furthermore, it was unknown whether glucose levels in canine vaginal fluid increased consistently during the reproductive cycle leading to ovulation. We tested every bitch in this clinical trial for vaginal fluid glucose levels daily, however these data were unable to be recorded accurately due to the inability of the glucometer used to detect the exact level of glucose below 20 mg/dL. This could be due to a limitation in the equipment used for this purpose (unable to detect glucose in vaginal fluid) or that the glucose may never have rose above the threshold that could be read. Therefore, it remains unclear whether glucose in the vaginal fluid of a bitch changes in relationship to the LH surge.

There is a possibility that it is not glucose that the LH strips are evaluating in the vaginal fluid or on the mucosa, but may be a similar monosaccharide, or steroid hormone that changes with the cycle, such as estradiol. Plasma estrogen concentrations rise and reach a peak level during proestrus leading into estrus. This surge of estrogen as it reaches this peak level is what triggers the LH surge, followed by ovulation about two days later [9].

We also conducted progesterone testing on every bitch about every other day to see when it reached a level that corresponded with the LH surge, close to 2 ng/mL. We know that serum progesterone rises prior to ovulation and reaches levels of around 5 ng/mL, but supplemental testing and information is required to accurately determine the optimal day for breeding. For example, on the first day of every trial, we took a vaginal cytology to see if there was significant cornification, a standard method of detecting estrus. According to our data, the positive Witness LH tests corresponded to the progesterone levels that are consistent with the LH surge, as was expected.

Because there have only been four bitches evaluated thus far, the data is inconclusive at this stage and will likely vary with a larger number of bitches. Initial data does not appear to support accuracy of these strips at predicting the day of the LH surge, however there does appear to be some

observation of color change 24-48 hours around the date of the surge. Sample collection is ongoing and statistical analysis will be conducted at the completion of the clinical trial.

It is a limitation to the study that the glucometer could not give an accurate reading of glucose levels in vaginal fluid, which leads us to speculate that there could be a different steroid hormone or compound that the strip is detecting, like estradiol, that is similar to the glucose monosaccharide structure. Additionally, the current limited sampling presents as another limitation to the study since further data will be needed to draw future conclusions about the efficacy of the LH strip.

CONCLUSION

This study was designed to provide beneficial and practical information for the purposes of breeding management. If effective, these strips could be a cheap and simple alternative for breeders, and provide a less stressful experience for the bitch, as vet visits and blood draws would be limited. Because this is still in progress and a larger sample size is needed, further research is required to gain a clearer understanding of the accuracy of the LH surge test strips, and their compatibility with the reproductive cycle of the domestic bitch. While we know that the strips respond to glucose, we do not yet see a consistent correlation throughout the periovulatory period, like it has been seen in women. Facing infertility and artificial insemination can be rather expensive, so coming up with an economical solution would be very appealing to owners.

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